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The Terminal Sialic Acid of Glycoconjugates on the Surface of Intestinal Epithelial Cells Activates Excystation of *Cryptosporidium parvum*▽

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The apicomplexan *Cryptosporidium parvum* reproduces in the intestinal epithelial cells of many mammalian species and is an agent of the important diarrheal disease cryptosporidiosis. Infection is transmitted fecal-orally by oocysts that pass through the stomach and excystation occurs in the intestine, releasing four invasive sporozoites. Some factors involved in inducing excystation have been identified, but the role of the enterocyte is not known. The present study showed that excystation was accelerated in the presence of the three enterocyte cell lines Caco2, HCT8, and CMT93. Epithelial cell lines derived from other organs, including the stomach, had no effect on excystation. No evidence was obtained that factors secreted from enterocytes induced excystation, but an enterocyte membrane preparation promoted sporozoite release. In addition, modification of the enterocyte surface by trypsin digestion or paraformaldehyde fixation abrogated the ability to enhance excystation. Importantly, the level of excystation in the presence of enterocytes decreased after treatment with either sialidase/neuraminidase to deplete surface terminal sialic acid or with lectins that specifically bind to sialic acid. Furthermore, the addition of sialic acid to oocysts in the absence of cells increased the level of excystation. These results suggest that sialic acid on the surface of enterocytes may provide an important local signal for the excystation of *C. parvum* sporozoites.

Cryptosporidium is a monoxenous parasite that develops in epithelial cells of a variety of host types, including mammals, birds, reptiles, and fish (16). Some cryptosporidia of mammals, e.g., *Cryptosporidium hominis*, have been reported to have strong host type preference, while *C. parvum* is able to infect many host species, including cattle and humans, and is an important zoonotic pathogen (14, 16). Infection with *C. parvum* may take place at various mucosal sites, although parasite development occurs principally in the intestine, causing enteritis in young animals and in humans (16). Symptoms may be more severe or even fatal in malnourished or immunocompromised hosts such as AIDS patients, and chemotherapeutic options are limited to a few drugs that have not always been beneficial (2).

The entire developmental cycle of *C. parvum*, comprising cycles of merogony, followed by gametogony and the formation of oocysts with four sporozoites, occurs within epithelial cells (6). Oocysts transmit infection in a fecal-oral manner either directly from host to host or indirectly through contaminated food or water (16). *Cryptosporidium* was originally assigned to the coccidia but the intracellular parasite has unique features, including its juxtaposition with the apical host cell membrane and isolation from the host cell cytoplasm (6). Indeed, ribosomal DNA sequence analysis has suggested the *Cryptosporidiidae* may be more closely related to the gregarines (3).

Attachment to and invasion of intestinal epithelial cells (enterocytes) by sporozoites of *C. parvum* involves specific host cell receptors and parasite ligand molecules that are secreted or expressed on the parasite surface (22). Excystation of the sporozoites preceding invasion takes place in the intestine, but the mechanisms leading to activation of sporozoites and opening of the oocyst wall have not been fully elucidated. Experimental investigations have suggested that efficient excystation requires a number of environmental triggers, including changes in temperature and pH, bile salts, and protease activity (20). Excystation is promoted by body temperature and enhanced by prior short-term exposure to acid, which may mimic the passing of oocysts through the stomach (7, 17). Both raised temperature and acidic conditions have been demonstrated to increase the permeability of the oocyst wall to small molecules (20). It has been established that bile salts increase the rate of excystation, and a study reported that, in addition, sodium deoxycholate improved the invasiveness of sporozoites for epithelial cells (8). Increased parasite protease activity during excystation has been described, and sporozoite release was hindered by protease inhibitors (9). The host protease trypsin, however, has appeared not to enhance the rate of excystation, although this enzyme increased the motility of sporozoites (17).

The possible involvement of enterocytes in excystation either through secreted factors or through direct contact with the oocyst has not been directly investigated. It has been reported that when *C. parvum* oocysts were brought into contact with enterocyte monolayers by centrifugation, the number of in-

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fected cells observed subsequently was greater (25). The reason for this was not determined, but improved excystation as a result of contact between oocysts and cell could not be discounted. The oocyst surface of *C. parvum* was demonstrated to be glycosylated (15), able to bind to lectins in a saccharide-specific manner (20) and have an adhesiveness to epithelial cell monolayers that can be inhibited by certain lectins (21). It is possible, therefore, that the host cell might provide development-related signals to the oocyst through direct contact. Indeed, lectin binding to oocysts increased excystation (24), suggesting that glycosylated oocyst surface molecules might be involved in inducing excystation.

The present investigation examined the hypothesis that direct contact between the epithelial cell surface and oocyst could provide a key stimulus for excystation. Human or mouse epithelial cell lines subjected to various treatments were cocultured with oocysts at 37°C. The results showed that excystation was strikingly increased with parasites in contact with enterocytes, whereas nonintestinal epithelial cells had no effect on sporozoite emergence. Further studies indicated that sialic acid expressed on the host cell surface served as an important signal for efficient *C. parvum* excystation.

MATERIALS AND METHODS

Parasite. Purified *C. parvum* oocysts of the IOWA isolate were purchased from Bunch Grass Farm, Idaho, and stored in phosphate-buffered saline (PBS) pH 7.2 at 4°C. In some experiments oocysts were surface sterilized by suspension in 10% (vol/vol) commercial bleach solution (sodium hypochlorite). The parasites were washed three times in PBS using a microcentrifuge at 13,000 rpm for 6 min before enumeration in a Neubauer hemacytometer.

Cell culture. Numerous epithelial cell lines from different mammals and organs were used: Caco2 (human colonic adenocarcinoma used between passage numbers 5 and 25), HCT8 (human ileocecal adenocarcinoma), RK13 (rabbit kidney), CMT93 (murine rectal adenocarcinoma), AGS (human gastric adenocarcinoma), and HeLa (human cervical carcinoma). Studies were also made with human primary intestinal mucosal myofibroblasts (provided by T. T. MacDonald, Barts and the London Medical School, Institute of Cell and Molecular Science, Centre for Infection) with ethical permission provided by the Queen Mary College, University of London Research Ethics Committee. The various cell types were grown in T-175 cm^2 flasks (VWR International Ltd) until confluent in a complete medium comprised of Dulbecco modified Eagle medium plus Glutamax supplemented with 10% heat-inactivated fetal calf serum, 10 mM L-glutamine, 1% nonessential amino acids, 100 U of penicillin/ml, and 100 μg of streptomycin/ml (all from Invitrogen) plus 50 mM HEPES buffer (Sigma-Aldrich). Culture took place in an incubator at 37°C with 5% CO_2 and 95% air. Adherent cells were released from the flask's surface using a working dilution of a trypsin-EDTA solution (Sigma-Aldrich). For the experiments, trypsin-treated cells (usually 10^5) were seeded into 24-well plastic tissue culture plates (Corning Costar) in 1 ml of medium and grown to confluence over 2 to 7 days depending on cell type.

Growing polarized Caco2 cells on filters. Caco2 cells were seeded at a density of 4×10^5/ml in polycarbonate culture plate inserts (0.4-μm pore size; Millipore) which were placed in six-well plates and incubated in 2 ml of culture medium placed on the filter insert and 2 ml of culture medium in the well. Formation of the polarized cell monolayer was determined by regular measurement of transepithelial electrical resistance (Millicell-ERS; Millipore). The monolayers were grown until the transepithelial resistance reading was over 1,000 Ω/cm^2 (4), which occurred by 13 to 21 days of culture.

Fixation of cell monolayers. Once cell monolayers in 24-well plates had grown to confluence, the culture medium was removed, and the wells were washed twice with PBS to remove any remaining traces of medium. Each monolayer was incubated with 100 μl of 1% paraformaldehyde at room temperature and then washed three times with PBS to remove any traces of paraformaldehyde. After fixation, the cells were examined microscopically to ensure the monolayers were intact.

Enzyme treatment of the epithelial cell surface. Cell monolayers were treated with either trypsin or neuraminidase (sialidase) to deplete the cell surface of proteins or terminal sialic acid residues. Prior to trypsin treatment the cells were washed with PBS twice and then incubated at 37°C with 0.25% trypsin in solution (Sigma-Aldrich) and examined every 2 to 3 min until they had partially rounded up but remained attached to the wells, which usually occurred by 8 min. At this point the trypsin was removed from the wells, and the cells were washed gently three times with PBS, taking care not to detach cells from the plastic surface. For neuraminidase treatment, the cells were incubated at 37°C with 2 mg of *Clostridium difficile* type V neuraminidase (Sigma-Aldrich)/ml in PBS for 15 min and then washed with medium.

Excystation of oocysts. Cell monolayers in 24-well plates had culture medium removed and were washed twice with PBS before the addition of 1×10^6 to 2×10^6 oocysts in 250 μl of serum-free medium (except where stated). As a control, oocysts were added to wells containing no cells. In some experiments, the bile salt sodium deoxycholate (0.1% [wt/vol]; Sigma-Aldrich) was added to the excystation medium. The plates were incubated at 37°C, and at particular time points the medium was removed from wells to Eppendorf tubes that were placed on ice to inhibit further excystation. Sporozoites (and in some experiments oocysts) from each sample were counted by using a Neubauer hemacytometer by microscopy with a ×40 objective lens.

Plasma membrane preparation. Caco2 cells were disrupted in a homogenization buffer (10 mM Tris-HCl, 1 mM EDTA, 200 mM sucrose [pH 7.4]), and the nuclei and cell debris were then removed from the homogenate by centrifugation at $900 \times g$ for 10 min at 4°C. The resultant supernatant was centrifuged at $110{,}000 \times g$ for 75 min at 4°C (Sorvall Th641) producing a membrane pellet. This pellet was then solubilized in buffer (10 mM Tris-HCl [pH 7.4], 1 mM EDTA, 0.5% Triton X-100) for a minimum of 1 h at 4°C. Any insoluble material was then extracted and removed by centrifugation at $14{,}000 \times g$ for 10 min at 4°C. The supernatant samples were stored at −70°C until required.

Lectins. A number of lectins at a concentration of 20 μg/ml in PBS were incubated with cells at 37°C for 60 min before the addition of oocysts. The lectins used and their saccharide specificities were as follows: concanavalin A (ConA; mannose and glucose), *Dolichos biflorus* agglutinin (DBA; *N*-acetylgalactosamine), peanut agglutinin (PNA; galactose); soybean agglutinin (SBA; *N*-acetylgalactosamine and galactose), *Ricinus communis* agglutinin I (RCA I; galactose or lactose), *Ulex europaeus* agglutinin (UEA I; fucose), wheat germ agglutinin (WGA; *N*-acetylglucosamine and sialic acid of some glycoproteins), *Sambucus nigra* agglutinin [SNA I; α(2,6)-linked sialic acid], and *Maackia amurensis* agglutinin [MAL; α(2,3)-linked sialic acid]. The cells were washed with medium before addition of oocysts.

Statistical analysis of results. In experiments four replicate samples were obtained for each treatment, and the results shown are representative of at least three experiments. The data were analyzed by using analysis of variance or Student *t* test when only two treatments were being compared.

RESULTS

Effect of Caco2 intestinal epithelial cells on the excystation of *C. parvum*. The ability of intestinal epithelial cells to influence the excystation of *C. parvum* was investigated by incubating oocysts with or without Caco2 monolayers in 24-well plates at 37°C. For comparison, the effect of a known stimulus of excystation, the bile salt sodium deoxycholate, was also studied. The salt concentration used was 0.1% (wt/vol) since higher concentrations damaged cell monolayers.

In controls, the level of excystation was low at 30 min and progressively increased subsequently until 120 min (Fig. 1). A similar pattern occurred when oocysts were incubated with sodium deoxycholate, although greater numbers of sporozoites were observed at these latter times compared to controls. Importantly, at 90 min samples incorporating Caco2 cells contained greater numbers of sporozoites than controls by a factor of >6-fold and samples with sodium deoxycholate without cells by 5-fold. There was a further increase in the yield of sporozoites (36%) when both bile salt and Caco2 cells were present compared to cells alone, indicating an additive effect of these stimuli. From 90 to 120 min there was a large reduction of sporozoites numbers in Caco2 samples. These results indicate that Caco2 cells had a potent ability to trigger excystation of *C.*

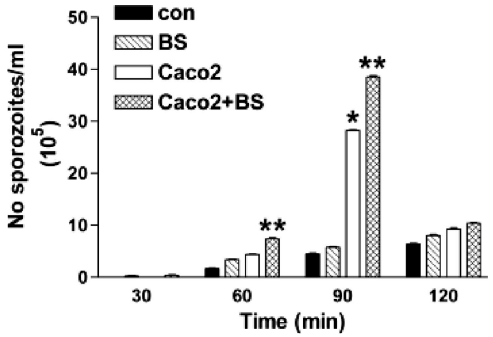

FIG. 1. Effect of the human Caco2 intestinal cell line and a physiologically compatible concentration of the bile salt sodium deoxycholate (BS) in triggering the excystation of sporozoites from oocysts of *C. parvum.* Oocysts were incubated at 37°C alone or with Caco2 and/or sodium deoxycholate, and the results are presented as mean values ± the standard deviation (SD) for sporozoites. Significant differences ($P < 0.0001$) were obtained for Caco2+BS compared to all other treatments (**) at 60 and 90 min and for Caco2 compared to BS and control treatments (*) at 90 min.

parvum and that this effect could be moderately increased when sodium deoxycholate was also added.

Ability of other intestinal and nonintestinal epithelial cells to influence excystation. In order to determine whether this excystation triggering property of Caco2 cells was shared by other types of epithelial cell, investigations were made with Caco2 and two other intestinal epithelial cell lines, HCT-8 and CMT93, as well as a nonintestinal epithelial cell line, RK13. After incubation at 37°C for 90 min, substantially higher numbers of sporozoites were observed in the presence of the intestinal Caco2, HCT8, and CMT93 cells compared to the control and, as before, the numbers of sporozoites had fallen by 120 min (Fig. 2A). Interestingly, at 90 and 120 min there were more sporozoites present when oocysts were cultured with CMT93 cells compared to Caco2 or HCT-8 cells. In contrast, incubation with the rabbit kidney cell line RK13 had no effect on the rate of appearance of sporozoites. This latter result led to the examination of other nonintestinal epithelial cell lines. Like RK13, the human epithelial cell lines AGS (from the stomach) and HeLa (from the cervix) had no effect on excystation. Similarly, contact between oocysts and human primary cells of intestinal myofibroblasts that in vivo are located immediately below the basolateral surface of epithelial cells did not affect excystation (see Fig. 4A for AGS; data not shown for the others).

It was possible that enterocytes prolonged the viability of sporozoites between 60 and 90 min rather than enhanced the rate of excystation. If that were the case, then the numbers of intact oocysts in the presence of Caco2 and RK13 cells should decrease at a similar rate. To examine this possibility, oocysts as well as sporozoite counts in the presence of these cell lines were measured during excystation (Fig. 2B). The patterns of sporozoite counts over time were similar to those observed above: with Caco2 cells a maximal value occurred at 90 min that was ninefold greater than that obtained with RK13 cells or controls. Coinciding with this peak there was a steep drop in the numbers of intact oocysts by a factor of 2.3; with four sporozoites per oocyst this increased level of excystation adequately explains the increased numbers of sporozoites at 90 min. This finding confirms that the increase in sporozoite numbers observed during incubation with intestinal epithelial cells represented increased excystation rather than prolonged sporozoite survival. The results of the above studies, therefore, showed that intestinal epithelial cells, but not epithelial cells

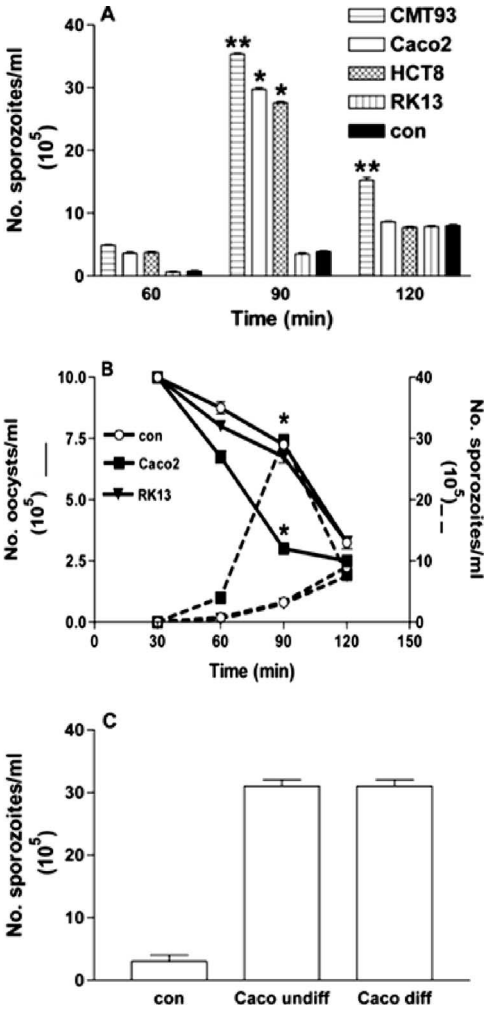

FIG. 2. Capacity of different epithelial cell lines to stimulate the excystation of *C. parvum* sporozoites. The results are presented as mean values ± the SD for sporozoite or intact oocyst numbers. (A) Excystation was examined in the presence of either human intestinal cells (Caco2 or HCT8), murine intestinal cells (CMT93), or rabbit kidney cells (RK13). At 90 min, significant differences in sporozoite numbers were obtained when intestinal cells were compared to kidney cells and control (*, $P < 0.0005$) or when CMT93 cells were compared to Caco2 and HCT8 cells (**, $P < 0.005$). At 120 min, the differences between numbers of sporozoites in the presence of CMT93 cells compared to other cells were also significant. (B) Measurement of sporozoite and intact oocyst numbers after incubation of oocysts in the presence or absence of Caco2 or RK13 cells. At 90 min, the numbers of intact oocysts and sporozoites in the presence of Caco2 cells were significantly different from those in the presence of RK13 cells and in the control samples (*, $P < 0.0001$). (C) Comparison of excystation in the presence of undifferentiated Caco2 cells grown for 4 days (undiff) and differentiated, i.e., polarized cells grown for 13 days (diff). After 90 min of incubation the numbers of sporozoites excysted in the presence of either cell type were not significantly different.

derived from other organs, specifically acted as a trigger for excystation of *C. parvum*.

In the experiments described thus far, nonpolarized, undifferentiated epithelial cells were used but in the intestine mature epithelial cells are polarized with the apical side bearing microvilli facing the gut lumen. Since Caco2 cells become polarized after extended culture, it was possible to determine whether differentiated cells could influence excystation. The cell monolayers were grown on tissue culture plate membrane inserts until the transepithelial resistance was >1,000 Ω/cm^2 (13 days), demonstrating that polarization had occurred (4). Polarization was confirmed by investigating expression of the brush border-specific enzyme sucrose isomaltase that is absent in undifferentiated enterocytes using reverse transcription-PCR (data not shown). As controls, cells were grown on membranes for only 4 days by which time they were confluent, but poorly differentiated, and so were similar to cells used in previous experiments. Measurement of excystation after incubation at 37°C for 90 min showed similar high numbers of sporozoites were obtained with each population of Caco2 cells (Fig. 2C). Hence, the ability of enterocytes to trigger excystation was retained when the cells had undergone polarization.

Roles of enterocyte secreted factors and plasma membrane in excystation. Enterocytes were likely to stimulate excystation of *C. parvum* via secreted factors or direct contact with oocysts and experiments were performed to examine each of these possibilities. The role of secreted factors was studied by comparing excystation in the presence of fresh culture medium, and similar medium removed from confluent CMT93 or RK13 cell monolayers after 48 h of incubation and referred to as "conditioned medium." Neither RK13 cells nor RK13-conditioned medium was able to influence excystation (Fig. 3A). Importantly, although the presence of CMT93 cells again stimulated excystation, exposure of oocysts to CMT93-conditioned medium did not. This indicated that host cell-secreted factors were unlikely to be important in activation of excystation and indirectly implied that direct contact between oocysts and the enterocyte surface was necessary.

Direct evidence that the enterocyte surface participated in triggering excystation was sought by incubation of oocysts with different concentrations of plasma membrane preparations of CMT93 cells. Figure 3B illustrates that 90 min of incubation with plasma membrane increased excystation levels in a concentration-dependent manner compared to when oocysts were incubated in medium alone. Optimal excystation was obtained with membrane protein at a concentration of 28 μg/ml. A similar result was obtained when Caco2 cell membrane preparations were used (results not shown). These findings support a role for an enterocyte membrane component(s) in excystation of *C. parvum*.

Further studies with CMT93 and RK13 cells investigated the effects on excystation of either treatment with trypsin to digest cell surface proteins or with paraformaldehyde to fix the cells. After treatment of RK13 or CMT93 and washing, oocysts were added and plates incubated at 37°C for 90 min. Neither paraformaldehyde nor trypsin treatment altered the inability of RK13 cells to influence excystation. In contrast, both paraformaldehyde and trypsin abrogated the boosting effect of CMT93 cells on excystation (Fig. 3C). This suggests that a component(s) of the intestinal epithelial cell membrane susceptible to fixation or trypsin digestion is responsible for facilitating excystation and that surface protein plays a key role in the mechanism.

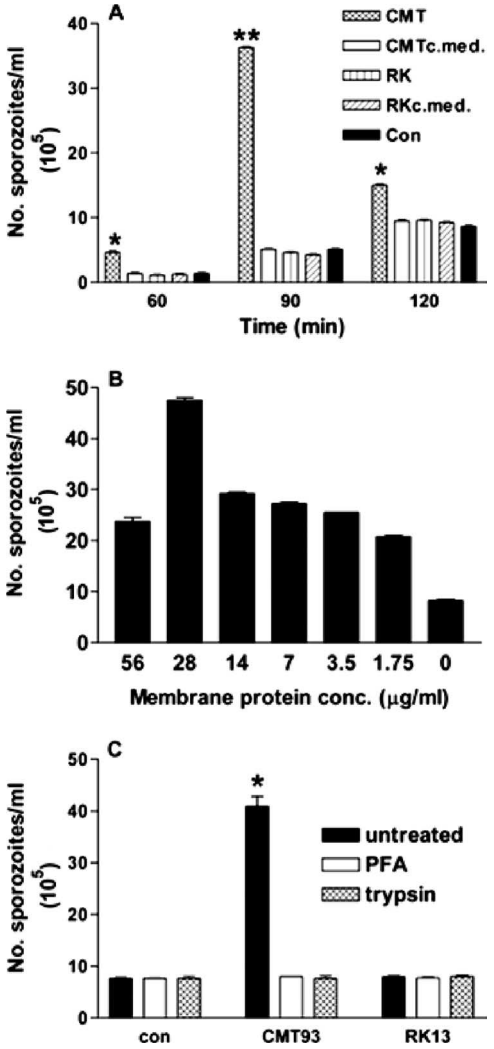

FIG. 3. Roles of enterocyte secreted factors and surface membrane in activating excystation of *C. parvum*. Each data point on graphs represents mean value ± the SD. (A) Excystation levels were measured in the presence of Caco2 or RK13 cells, and fresh medium or conditioned medium (c.med.) was removed from these cell lines after 48 h culture. There was a significant difference between sporozoite numbers in the presence of Caco2 cells compared to other groups (**, $P < 0.0001$; *, $P < 0.001$), but there were no significant differences between the excystation levels in Caco2 or RK13 cell conditioned medium and in fresh medium. (B) Effect of a CMT93 cell membrane preparation on excystation. Oocysts were incubated for 90 min at 37°C with different concentrations of membrane expressed as protein content. The presence of membrane significantly affected sporozoite release in a dose-dependent manner ($P < 0.0001$). (C) Effect on excystation of modification of the CMT93 or RK13 membrane by paraformaldehyde (PFA) fixation or trypsinization. Both treatments significantly affected excystation triggering by CMT93 cells (*, $P < 0.0001$).

Involvement of sialic acid of enterocyte glycosylated surface molecules in excystation. Glycosylated molecules on host cell surfaces play an important part in interactions with pathogenic microorganisms. The role of N-linked glycosylated molecules

in excystation was investigated by culturing Caco2 cells for 4 days with tunicamycin, which inhibits this type of glycosylation (13). However, this treatment had no effect on the ability of the cells to induce excystation (data not shown), suggesting that only O-linked glycosyl groups were involved in excystation.

Terminal sialic acid in glycosylated host cell surface molecules are often involved in attachment and invasion of microbial pathogens (19). The role of α(2,3)- and α(2,6)-linked sialic acid in excystation was studied by using a sialidase/neuraminidase which cleaves both linkages. Cell monolayers of Caco2 or the human gastric epithelial cell line AGS were incubated with 2 mg of neuraminidase/ml for 15 min (a treatment previously shown to deplete epithelial cell surface sialic acid [12]), washed, and then oocysts were added before incubation for 90 min at 37°C. The AGS cells, like RK13 cells previously, did not influence excystation whether treated with enzyme or not (Fig. 4A). Significantly, after exposure of Caco2 cells to neuraminidase, the number of sporozoites released at 90 min was reduced by 45%. This observation indicated that sialylated glycans of the enterocyte surface played an important part in initiating excystation. A further experiment examined the effect on the excystation-inducing capacity of CMT93 after incubation with various lectins that exhibit binding affinity for specific monosaccharides (see Materials and Methods). Some lectins had no effect on excystation (DBA, RCA I, and UEA I), while others reduced the excystation level moderately by 24 to 28% (ConA, PNA, and SBA) (Fig. 4B). The lectins with specificity for sialic acid (WGA, SNA I, and MAL), however, inhibited excystation to a greater extent, by 55 to 57%. SNA I and MAL have strong specificity for terminal α-(2,6)- and α-(2,3)-linked sialic acid, respectively, so these observations imply that each configuration on the surface of enterocytes may act as an important trigger for the excystation of *C. parvum*.

In view of these results, it was of interest to establish whether in the absence of enterocytes the addition of sialic acid to oocysts might affect the rate of excystation. After 90 min of incubation with oocysts, exogenous sialic acid had increased the number of sporozoites in a concentration-dependent manner (Fig. 4C). The optimal concentration of sialic acid for excysation, in the range 0.13 to 3.2 mM, increased sporozoite numbers sixfold compared to the control. These results confirm that sialic acid is an important trigger for excystation of *C. parvum*.

DISCUSSION

Excystation of *C. parvum* is promoted by host ambient temperature, parasite proteases, and gastrointestinal luminal factors such as stomach acid and bile salts (20). The role of intestinal epithelial cells infected by the parasite in excystation has not been investigated previously. The present findings suggest that direct contact between oocysts and enterocytes provides an additional significant stimulus for excystation and that terminal sialic acid residues of cell surface glycoproteins play a major part in the process.

Coculture of oocysts with Caco2 cell monolayers in 24-well plates at 37°C accelerated the emergence of sporozoites from oocysts, with a peak in sporozoite numbers occurring at around 90 min. Coinciding with this peak was a sharp decrease in numbers of intact oocysts, confirming that the cells were en-

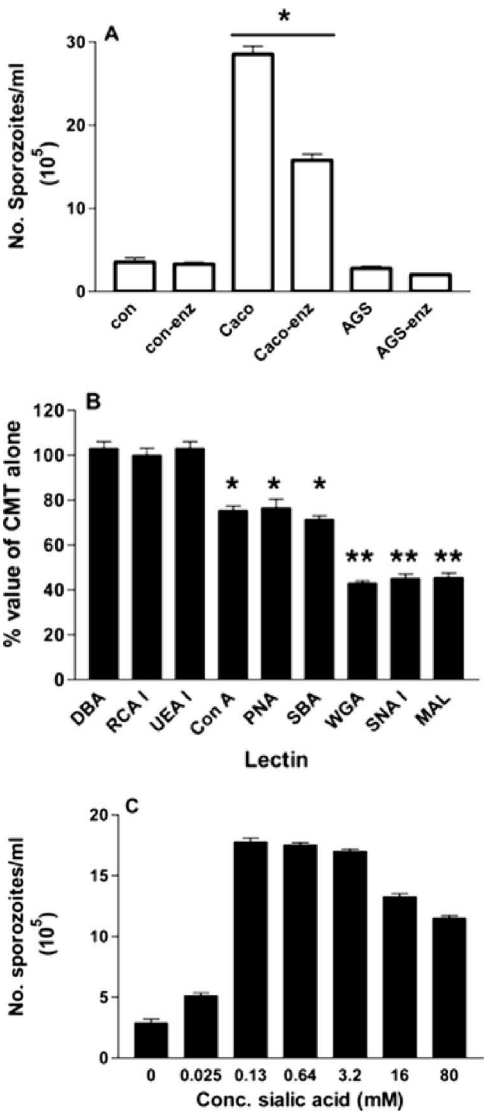

FIG. 4. Involvement of sialic acid in inducing *C. parvum* excystation. Excystation was measured after 90 min of incubation of oocysts while exposed either to epithelial cells treated to remove or block surface sialic acid or exogenous sialic acid. (A) Caco2 and AGS cells were treated with 2 mg of neuraminidase/ml for 15 min to deplete surface sialic acid prior to the addition of oocysts, and subsequently measurements were made of sporozoite mean values ± the SD. Neither untreated nor enzyme treated AGS cells (AGS-enz) had a significant effect on excystation. Treatment of Caco2 cells with enzyme (Caco-enz) significantly affected the capacity of the cells to stimulate sprozoite release (*, $P < 0.005$). (B) Effect of blocking carbohydrate molecules on the CMT93 cell surface with lectin on the ability of the cells to stimulate excystation. The sporozoite release data are presented as a percentage of the mean value ± the SD for CMT93 cells that were not treated with lectin. The binding to enterocytes of DBA, RCA I, and UEA I had no significant effect on excystation. One group of lectins—ConA, PNA, and SBA—had a significant effect on excystation (*, $P < 0.005$), while another group with specificity for sialic acid—WGA, SNA I, and MAL—had an even greater effect (**, $P < 0.0001$). (C) Measurement of excystation in the absence of cells but with different concentrations of exogenous sialic acid added to the medium. There was a significant concentration-dependent effect of sialic acid on sporozoite release ($P < 0.001$).

hancing excystation rather than preserving the viability of released sporozoites. In most experiments nonpolarized, i.e., undifferentiated, enterocytes were used, but when the Caco2 cells were grown to induce polarization, similar results were obtained, indicating that the state of differentiation of the host cells did not affect the ability to induce excystation.

Bile salts are commonly used as a component of in vitro excystation media (20), but Caco2 cells were more effective in activating excystation than the bile salt sodium deoxycholate used at a concentration that could be used under physiological conditions. Excystation in the presence of both bile salt and Caco2 cells, however, was significantly better than in samples containing cells only. Bile salts may have a separate major role in parasite infection by activating sporozoite gliding required for host cell invasion (8).

Two other enterocyte cell lines, one human (HCT8) and one murine (CMT93), were also able to enhance excystation. CMT93 was more effective than either HCT8 or Caco2, but the reason for this is unknown. A significant observation was that epithelial cell lines derived from the human cervix, rabbit kidney, and human stomach did not increase excystation, implying that the stimulating capacity of epithelial cells might be restricted to enterocytes.

It is likely that enterocytes would stimulate excystation either by secreted factors or as a result of direct contact. The rate of excystation in the presence of conditioned cell culture medium that had supported a confluent enterocyte cell line for 48 h was no better that that obtained with fresh medium, suggesting that a secreted product was unlikely to be important for triggering excystation. This implied that direct contact with the enterocyte surface might be required and three observations supported this possibility. First, soluble enterocyte cell membrane preparations were able to increase the rate of excystation. Second, excystation was not stimulated by enterocytes subjected to trypsin digestion, indicating that surface protein played a major part in inducing excystation. Third, when enterocytes were exposed to the fixative paraformaldehyde the cells lost the ability to enhance excystation. Paraformaldehyde treatment of enterocytes does not impair the receptor site for *C. parvum* sporozoite attachment (10), so the loss of function in excystation might be associated with cross-linking of surface molecules in fixation. Interestingly, surface sterilization of oocysts prior to experiments using dilute sodium hypochlorite did not reduce the capacity of enterocytes to enhance excystation (data not shown), although the surface molecules affected by this treatment are not known.

Host cell surface glycosylated molecules containing terminal sialic acids can be important targets in the pathogenesis of microbial infections (5, 19). Commonly, this sugar is linked to other sugars in α(2,3)- or α(2,6)-linked configurations (1, 18). In Caco2 cells terminal sialic acid is distributed uniformly across the surface, although in polarized cells the α(2,3)-linkages may be more concentrated apically (23). Significantly, depletion of terminal α(2,3)- and α(2,6)-linked sialic acid from the enterocyte surface using sialidase/neuraminidase prior to the addition of oocysts reduced the level of excystation induced by the cells. Similarly, blocking access to enterocyte surface sialic acid by exposure to sialic acid-binding lectins hampered the capacity of the cells to induce excystation more than lectins specific for other monosaccharides. The lectins MAL and SNA I binding α(2, 3)- and α(2, 6)-linkages, respectively, were equally effective in inhibiting induction of excystation. These findings indicated that enterocyte surface sialoglycoconjugates were important for triggering excystation. The result showing that tunicamycin-treated enterocytes were not impeded in stimulating excystation suggested also that O-linked but not N-linked glycoconjugates were involved in the induction of excystation (13). Further examination of the role of sialic acid indicated that, in the absence of cells, excystation was substantially enhanced by the addition of this monosaccharide to the medium. These observations, therefore, demonstrate an important role for sialic acid in activating excystation.

Terminal sialic acid is a key component of the erythrocyte surface receptor for *Plasmodium falciparum* merozoites since its removal prevents parasite attachment to the cell (11). *Trypanosoma cruzi* requires a sialoglycoconjugated surface protein to invade host cells and must acquire the sialic acid enzymatically from host molecules (26). Bacterial pathogens may express sialidases to cleave sialic acid from the host cell surface either for nutritional purposes or to provide a binding site on the cell (19).

The mechanism by which host cell sialic acid influences excystation of *C. parvum* is unclear at present. The sugar might act as an exogenous source of nutrition for the sporozoite and help to activate the parasite. That being the case, however, it is not clear why sporozoite numbers decreased soon after excystation, although this might have been in part due to rapid infection of the cells. A nutritional role would probably require the involvement of a parasite sialidase, but excystation in the presence of cells was unaffected by the introduction of the sialidase inhibitor 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (results not shown). An alternative mechanism for activating excystation fitting the observations involves the oocyst surface possessing a molecular sensor for particular sialoglycoconjugates. On ligation, the sensor molecule would transduce a signal to the oocyst interior, leading to sporozoite activation. In relation to this, it has been reported by others that exposure of oocysts to certain lectins increased the level of excystation (24).

Why only intestinal epithelial cells were able to stimulate excystation of *C. parvum* is unknown. The surface of other types of epithelial cells are as rich in sialic acid as enterocytes (23), so the most likely explanation is that there are enterocyte-specific sialoglycoconjugated proteins involved in excystation. Our results are consistent with the intestine being the predominant and preferred location for *C. parvum* development and, therefore, a signal for excystation from the indigenous host cell would be advantageous to parasite survival. It is important, however, to differentiate between the capacities of epithelial cell types to induce excystation and support parasite development since, in vivo, *C. parvum* can reproduce at extraintestinal mucosal sites (16), while, in vitro, the parasite develops in nonintestinal epithelial cell lines such as MDCK that express receptors for sporozoite attachment (10).

Two final points can be made in relation to the present study. First, the fact that neither enzymatic depletion nor lectin blocking of sialic acid could abrogate the stimulatory effect of enterocytes on excystation suggests that the cells might stimulate excystation by other, as-yet-unknown mechanisms. Sec-

ond, it would be of interest to learn whether mucin that is rich in sialic acid can also activate excystation.

In conclusion, the present study has identified a novel trigger for excystation of *C. parvum* involving contact between the oocyst and enterocyte surface sialic acid. Further investigations of mechanisms that interfere with *C. parvum* excystation in the intestine might lead to novel prophylactic approaches in cryptosporidiosis research.

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